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Abstract: Erythropoietin (Epo) treatment has been shown to induce mitochondrial biogenesis in cardiac muscle along with enhanced mitochondrial capacity in mice. We hypothesized that recombinant human Epo (rhEpo) treatment enhances skeletal muscle mitochondrial oxidative phosphorylation (OXPHOS) capacity in humans. In six healthy volunteers rhEpo was administered by sub-cutaneous injection over 8 weeks with oral iron (100 mg) supplementation taken daily. Mitochondrial OXPHOS was quantified by high-resolution respirometry in saponin-permeabilized muscle fibers obtained from biopsies of the vastus lateralis before and after rhEpo treatment. OXPHOS was determined with the mitochondrial complex I substrates malate, glutamate, pyruvate, and complex II substrate succinate in the presence of saturating ADP concentrations, while maximal electron transport capacity (ETS) was assessed by addition of an uncoupler. rhEpo treatment increased OXPHOS (from 92 ± 5 to 113 ± 7 $\text{pmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$) and ETS (107 ± 4 to 143 ± 14 $\text{pmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$, $p < 0.05$), demonstrating that Epo treatment induces an upregulation of OXPHOS and ETS in human skeletal muscle.

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Erythropoietin treatment enhances muscle mitochondrial capacity in humans

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Erythropoietin (Epo) treatment has been shown to induce mitochondrial biogenesis in cardiac muscle along with enhanced mitochondrial capacity in mice. We hypothesized that recombinant human Epo (rhEpo) treatment enhances skeletal muscle mitochondrial oxidative phosphorylation (OXPHOS) capacity in humans. In six healthy volunteers rhEpo was administered by sub-cutaneous injection over 8 weeks with oral iron (100 mg) supplementation taken daily. Mitochondrial OXPHOS was quantified by high-resolution respirometry in saponin-permeabilized muscle fibers obtained from biopsies of the vastus lateralis before and after rhEpo treatment. OXPHOS was determined with the mitochondrial complex I substrates malate, glutamate, pyruvate, and complex II substrate succinate in the presence of saturating ADP concentrations, while maximal electron transport capacity (ETS) was assessed by addition of an uncoupler. rhEpo treatment increased OXPHOS (from 92 ± 5 to 113 ± 7 pmol·s⁻¹·mg⁻¹) and ETS (107 ± 4 to 143 ± 14 pmol·s⁻¹·mg⁻¹, $p < 0.05$), demonstrating that Epo treatment induces an upregulation of OXPHOS and ETS in human skeletal muscle.

Keywords: mitochondria, oxidative phosphorylation, rhEPO, muscle, humans

INTRODUCTION

Erythropoiesis is regulated through activation of the erythropoietin (Epo) receptor (Epo-R) present on erythroid progenitor cells stimulating growth and differentiation of the red blood cell precursors (Jelkmann, 1992). In addition to this, Epo also suppresses plasma volume and thereby effectively controls arterial content (Lundby et al., 2007; Olsen et al., 2011). Epo-R have been observed in several non-hematopoietic tissues and cells such as the liver, pancreas, heart, uterus, central nervous system, vascular endothelium, satellite cells, and skeletal muscle (Farrell and Lee, 2004; Lundby et al., 2008a; Rundqvist et al., 2009; Carraway et al., 2010). The functionality of the Epo-R in mediating the effect of Epo in non-hematopoietic cells is currently debated (Sinclair et al., 2010), however Epo treatment in the mouse has been found to induce mitochondrial biogenesis in cardiac and skeletal muscle accompanied by an increase in state 3 mitochondrial respiratory capacity (Carraway et al., 2010). Consistent with this pattern, mice electrotransfected with Epo gain less body fat when on a high fat diet as compared to matched control mice (Hojman et al., 2009), and there is some evidence for a shift in skeletal muscle fiber type in mice with Epo treatment (Cayla et al., 2008). In humans no effect on skeletal muscle capillarization, fiber type (Lundby et al., 2008a), and myoglobin (Robach et al., 2009) have been found with rhEpo treatment. Yet, the finding that recombinant human Epo (rhEpo) enhances submaximal endurance performance to a greater extent (~54%) than what can be accounted for by increased blood oxygen carrying capacity (~12%), and that lactate is lower at a similar relative submaximal exercise intensity after rhEpo treatment

(Thomsen et al., 2007), suggests an additional effect of rhEpo at the metabolic level. The purpose of the present study was hence to test the hypothesis that rhEpo treatment leads to enhanced skeletal muscle mitochondrial oxidative phosphorylation capacity (OXPHOS) in humans.

MATERIALS AND METHODS

SUBJECTS

Six healthy young men aged 21 ± 2 years, height 180 ± 1 cm, weight 73 ± 2 kg, volunteered to participate in the study. None of the volunteers had been exposed to rhEpo previously. All subjects agreed to maintain their level of physical activity unchanged throughout the trial period. Activity level was monitored throughout the study period with a heart rate monitor (Polar Electro, Finland) worn at all hours of the day except when sleeping. Subjects were informed about the possible risks and discomfort involved before giving their written consent to participate. The study was carried out according to the Declaration of Helsinki and was approved by the Ethical committee of Copenhagen (H-2-2010-104).

RECOMBINANT HUMAN EPO TREATMENT

The rhEpo treatment aimed to increase the hematocrit (Hct) to ~50% and to maintain that value throughout the study period. The value of 50% was chosen, partly from a health perspective to minimize the risk of thrombosis and partly because it is the maximum allowed value in many sports. We treated the subjects with the following regime: 1 week of daily sub-cutaneous (SC)

injections of 5000 IU of rhEpo (NeoRecormon Roche, Schweiz), followed by 1 week without treatment. Thereafter the subjects were given one weekly SC injection for 7 weeks, titrating the rhEpo dosage (**Figure 1**) depending on their Hct level (giving either 0, 2500, or 5000 IU). The subjects were instructed to take daily iron supplements of 100 mg throughout the study period, starting 2 weeks prior to the rhEpo treatment. Venous blood was drawn to assess blood hematocrit (Radiometer, ABL 800, Denmark) weekly to monitor the Hct value over the course of rhEpo treatment.

PEAK OXYGEN CONSUMPTION

Expired minute ventilation, O_2 fraction, and pulmonary $\dot{V}O_2$ were measured continuously using an automated metabolic cart (Oxycon Pro, Jaeger, Germany). Subjects performed exercise on an electrically braked cycle ergometer (Ergometrics 800, Jaeger, Germany). The exercise protocol began with a 6-min warm up period at 60 W followed by incremental increases in workload by 35 W each minute until exhaustion.

BODY COMPOSITION (DXA)

Dual X-ray absorptiometry (DXA) scans were performed before and after the study period for determination of percentage of body fat and lean body mass (Lunar, Madison, WI, USA). Fat free mass and fat mass were expressed as absolute values.

SKELETAL MUSCLE BIOPSIES

A muscle biopsy was taken prior to the 8 weeks of rhEpo treatment and again 1 week after the treatment period ended. After local anesthesia (1% lidocaine) of the skin and superficial muscle fascia, the biopsy was obtained from the vastus lateralis muscle using the Bergström method. A portion of the obtained muscle tissue was frozen immediately in liquid nitrogen and stored at -80°C for later analysis of citrate synthase (CS) as a mitochondrial matrix marker reflecting mitochondrial content. A small portion of the biopsied muscle was placed in relaxing medium (10 mM Ca-EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM

K-MES, 0.5 mM DTT, 6.5 mM MgCl_2 , 5.8 mM ATP, 15 mM phosphocreatine, pH 7.1) at $2-4^\circ\text{C}$. Individual fiber bundles were then separated with forceps to achieve a high degree of fiber separation. The fiber bundles were then permeabilized for 30 min in 3 ml of ice-cold relaxing medium with saponin ($50\text{ }\mu\text{g}\cdot\text{ml}^{-1}$). After chemical permeabilization, the tissue was rinsed twice for 10 min in chilled solution of EGTA (0.5 mM), $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ (3 mM), K-lactobionate (60 mM), Taurine (20 mM), KH_2PO_4 (10 mM), HEPES (20 mM), sucrose (110 mM), BSA (1 g/l), at pH 7.1. The muscle bundles were then blotted and measured for wet weight in a precision balance. The muscle fibers were then transferred immediately into the respirometer (Oxygraph-2k, Oroboros Instruments, Austria) containing respirometry medium at ambient air saturation at a chamber temperature of 37°C .

RESPIROMETRY

Standardized instrumental and chemical calibrations were undertaken on the Oxygraph-2k high-resolution respirometer and applied to correct for back-diffusion of oxygen into the chamber, leak from the exterior, oxygen consumption by the chemical medium and O_2 sensor. O_2 flux was resolved by software (Dat-Lab 4, Oroboros Instruments, Austria) that calculates non-linear changes in the negative time derivative of the oxygen concentration signal.

All muscle respirometry measurements were done in duplicate. Oxygen diffusion limitation of respiration was prevented by maintaining oxygen levels in the respirometer above air saturation (450–300 nM range). A substrate-inhibitor titration protocol was applied as follows. Routine respiration (state 2; absence of adenylates) with electron supply of NADH to Complex I was assessed by addition of malate ($2\text{ mmol}\cdot\text{l}^{-1}$), followed by titration of pyruvate ($10\text{ mmol}\cdot\text{l}^{-1}$), and glutamate ($10\text{ mmol}\cdot\text{l}^{-1}$). Coupled state 3 respiration of Complex I was achieved by addition of saturating adenosine-di-phosphate (ADP $5\text{ mmol}\cdot\text{l}^{-1}$). Addition of succinate ($10\text{ mmol}\cdot\text{l}^{-1}$) then stimulated respiration further through convergent electron input by FADH_2 to the Q-junction through complex II to achieve maximal coupled respiration (OXPHOS). Intactness of the outer mitochondrial membrane was tested by quantifying respiration after titration of cytochrome *c* ($10\text{ }\mu\text{mol}\cdot\text{l}^{-1}$). Uncoupled respiration and maximal electron transport capacity (ETS) was assessed by addition of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) in steps of 0.25 mM. The isolated activity of cytochrome oxidase (COX) was measured by addition of N,N,N',N'-tetramethyl-1,4-benzenediamine, dihydrochloride (TMPD $500\text{ }\mu\text{M}$) + ascorbate (2 mM) after antimycin A ($2.5\text{ }\mu\text{M}$).

CITRATE SYNTHASE ACTIVITY

Skeletal muscle tissue was freeze-dried and dissected free of all visible blood, adipose, and connective tissue under a stereomicroscope. Approximately 2 mg of the dissected tissue was homogenized (TissueLyser II, Qiagen, Germany) in $800\text{ }\mu\text{l}$ 0.3 M K_2HPO_4 , 0.05% BSA, pH 7.7. Spectrometric determination of CS activity was performed at 25°C by 50 times dilution in a solution containing 100 μM acetyl-CoA, 0.5 mM NAD (free acid), 1 mM sodium malate, 8 $\mu\text{g}\cdot\text{ml}^{-1}$ malate dehydrogenase ($1200\text{ U}\cdot\text{mg}^{-1}$, Boehringer Germany), 2.5 mM EDTA, 10 mM Tris-HCl (pH 8.0).

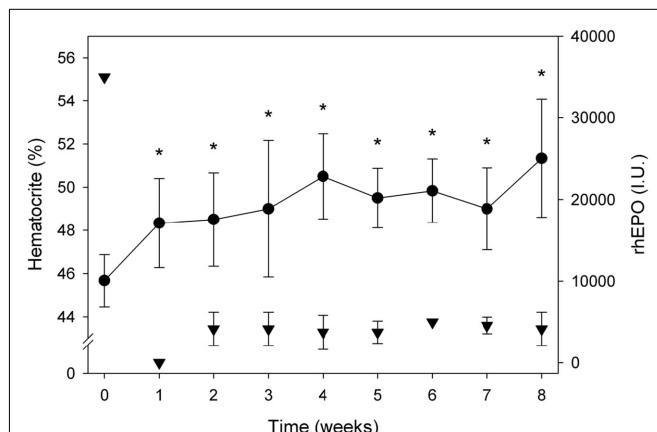


FIGURE 1 | Blood hematocrit (%) and rhEPO dosage over the 8 week treatment period. Data are means \pm SEM. Asterisk * indicates significant difference pre vs. post, $p < 0.05$.

DATA ANALYSIS

To analyze regulatory characteristics of specific branches of the ETS, several ratio comparisons were made. Physiological Complex I + II substrate combinations support maximum ETS and OXPHOS capacities due to the additive effect of multiple electron supply pathways converging at the Q-junction. The substrate control ratio (SCR) calculated as the ratio of Complex I to Complex I + II respiratory rate (GMP3/GMPS3) was determined for analysis of the substrate limitation of complex I respiration to maximal coupled mitochondrial respiration. The non-coupled state of maximum respiration (E) was experimentally induced by titration of the uncoupler FCCP to collapse the proton gradient across the mitochondrial inner membrane and thus to measure the capacity of the ETS in the open-circuit operation of respiration. The ratio *P/E* indicates the extent of limitation of ETS by the phosphorylation system, where a ratio of 1.0 indicates no limitation. The isolated activity of COX measured with ascorbate and TMPD after ETS inhibition by antimycin A was taken as a functional measure of COX enzyme activity in the intact system. All values are given as means \pm SEM for all experiments. A one-way ANOVA was used to detect differences over time during the Epo treatment period. Paired *t*-tests were used to determine differences in mitochondrial OXPHOS and ETS capacity pre-post Epo treatment. For all evaluations, a *p* value of <0.05 was considered significant.

RESULTS

BLOOD AND EXERCISE RESPONSES

The mean Hct of the six subjects before rhEpo treatment was $45.7 \pm 0.5\%$, increasing to $48.3 \pm 0.8\%$ within 1 week with the 35000 IU dose of rhEpo. After the 8 week intervention period, the mean Hct for the whole group was increased significantly to $51 \pm 1\%$ (Figure 1). Three of the six subjects received full treatment dosage with a total of 70,000 IU rhEpo over 8 weeks, while two subjects were administered 67,500 and 62,500 IU, respectively. The remaining subject exhibited a strong response to the rhEpo injections, and received a total of 50,000 IU. Epo treatment increased $\dot{V}O_2$ max by $8.3 \pm 2\%$ from 54 ± 3 to 58 ± 3 ml·kg⁻¹·min⁻¹ (Table 1). Body composition expressed as BMI and body fat% was unchanged by rhEpo treatment (Table 1). Compliance to stable activity level during the treatment period was reflected in stable daily and weekly average HR, results not shown.

MITOCHONDRIAL FUNCTION

After Epo treatment state 3 respiration with Complex I substrates (MPG3) was 52 ± 5 before and 64 ± 7 pmol·s⁻¹·mg⁻¹.

Table 1 | Subject characteristics at baseline and after 8 weeks of rhEpo treatment.

	Pre	Post
Weight (kg)	73 \pm 2	73 \pm 2
BMI	23 \pm 1	22 \pm 1
Body fat (%)	19 \pm 2	17 \pm 3
$\dot{V}O_2$ max (ml·kg ⁻¹ ·min ⁻¹)	54 \pm 3	58 \pm 3*

Data are means \pm SEM.

*Indicates significant difference pre vs post.

after rhEpo while respiratory rate for convergent electron supply through the Q-junction for Complex I + II (Figure 2) increased from 92 ± 6 to 113 ± 7 pmol·s⁻¹·mg⁻¹. The substrate control ratio (MPG3/MPGS3) was unchanged (0.56 ± 0.04 vs. 0.56 ± 0.03) by Epo treatment (Table 2), while the maximal ETS measured with FCCP titration increased from 107 ± 4 to 143 ± 13 pmol·s⁻¹·mg⁻¹. The coupling (phosphorylation) control ratio (*P/E*) reflecting the ratio of OXPHOS to ETS was unchanged (from 0.87 ± 0.04 to 0.83 ± 0.03), indicating similar control of ETS capacity by the OXPHOS system (Table 2). Some of the samples were excluded from statistical analysis due to a high stimulation of respiration by addition of cytochrome c. In the baseline experiments one sample showed an increase of respiration of 26% and in the post Epo experiments 2 samples in different subjects were excluded with responses of 40 and 60% respectively. For these subjects a single respirometric measurement was used in the analysis. The isolated activity of COX was similar before and after (333 ± 25 vs. 320 ± 20 pmol·s⁻¹·mg⁻¹) Epo treatment, as was CS activity (129 ± 10 vs. 139 ± 13 μ mol·g⁻¹·min⁻¹, *p* = 0.06).

DISCUSSION

The main finding in this study is that rhEpo treatment in humans increased skeletal muscle mitochondrial respiratory capacity. OXPHOS capacity with substrates providing electrons to complex I and II of the electron transport chain was elevated by 22% with rhEPO treatment (Figure 2). All subjects exhibited an increase in OXPHOS with Epo which represents the maximal ADP-stimulated oxygen consumption linked to the formation of ATP. The increase in whole body $\dot{V}O_2$ peak (8%) was similar to the increase in blood hematocrit (12%) as found previously (Lundby et al., 2008b), but much smaller than the increase in muscle oxidative capacity.

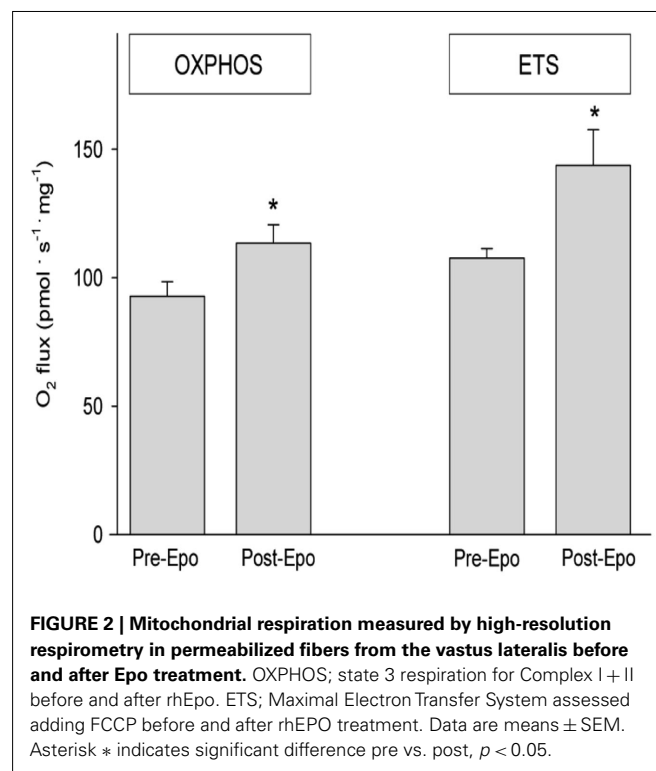


Table 2 | Mitochondrial flux control ratios before and after rhEpo treatment.

	Pre	Post
MPG3/MPGS3	0.56 ± 0.04	0.56 ± 0.03
P/E	0.87 ± 0.04	0.83 ± 0.03
P/CS	0.73 ± 0.07	0.83 ± 0.06
P/COX	0.28 ± 0.01	0.38 ± 0.04*
E/COX	0.33 ± 0.02	0.45 ± 0.06*

MPG3/MPGS3 (*M*, malate, *P*, pyruvate, *G*, glutamate, *S*, succinate, 3=ADP) = substrate control ratio for Complex I: Complex I+II respiration; P/E = phosphorylation control ratio indicating the ratio of state 3 respiration to maximal electron transport capacity determined by addition of the uncoupler FCCP; P/CS = OXPHOS relative to citrate synthase activity; COX = isolated Vmax of cytochrome oxidase measured in the presence of TMPD+ascorbate and Antimycin A; P/COX = the ratio of coupled state 3 respiration to COX activity; E/COX = the ratio of ETS to COX activity. Data are means ± SEM.

*Indicates significant difference pre vs post.

This finding is consistent with the concept that humans have an excess capacity of muscle mitochondria over O₂ delivery due to limits of cardiac output during whole body exercise (Boushel et al., 2011). In that study peak VO₂, cardiac output, limb blood flow, a-v femoral blood gases, O₂ diffusion capacity and muscle mitochondrial capacity were measured. For O₂ delivery to match muscle mitochondrial capacity during cycling exercise a blood flow increase of ~8 l min⁻¹ at peak exercise would be required. The finding of a 22% increase in mitochondrial OXPHOS in the present study indicates that the disparity between O₂ delivery and muscle oxidative capacity is even larger with Epo treatment, and the proportional increase in hematocrit and whole body VO₂ emphasizes the large dependence on O₂ delivery.

The *non-coupled* state of maximum respiration was experimentally induced by titration of the uncoupler FCCP for measurement of the capacity of the ETS, and this O₂ flux rate was also increased after rhEpo treatment. Proportional enhancement of the OXPHOS system was reflected in a similar ratio of Complex I vs. complex I + II respiration (MPG3/MPGS3; Table 2). These findings are consistent with the higher mitochondrial respiratory capacity reported in cardiac muscle of mice (Carraway et al., 2010). That study demonstrated that Epo treatment activates the JAK/STAT, protein kinase B, and eNOS pathways, leading to upregulation of nuclear respiratory factor-1, peroxisome proliferator-activated receptor-coactivator 1 and mitochondrial transcription factor-A gene expression. The Epo-R was required for this response, since Epo-R silencing in cardiomyocytes blocked the Epo-mediated nuclear translocation of nuclear respiratory factor-1. A similar but modest activation of the JAK/STAT pathway by Epo has been demonstrated in human skeletal muscle following acute exercise (Rundqvist et al., 2009).

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- We measured the isolated activity of COX as an independent measure of mitochondrial functionality by titrating the redox substrates ascorbate and TMPD following inhibition of the respiratory chain upstream at Complex III with Antimycin A. We found no change in COX activity with rhEpo treatment. Relative to the changes in OXPHOS and ETS, this indicates qualitative changes in mitochondrial function. Traditionally it has been thought that increases in mitochondrial respiratory capacity result from an enlargement of mitochondrial volume rather than by altered intrinsic capacity of mitochondria (Holloszy, 1967; Reichmann et al., 1985; Tonkonogi and Sahlin, 1997; Tonkonogi et al., 1997, 2000). A generally held view is that mitochondrial respiratory capacity is relatively constant at ~5 ml O₂ per milliliter of cristae volume (Schwermann et al., 1989). However, more recent studies have shown that different lifestyle influences such as acute exercise (Fernström et al., 2004) and high fat diet (Anderson et al., 2009; Skovbro et al., 2011) may change the qualitative properties of mitochondria. This has also been shown in pathological conditions such as type 2 diabetes (Mogensen et al., 2007; Phielix et al., 2008) and chronic obstructive lung disease (Naimi et al., 2011). The intrinsic properties of the mitochondrion are usually determined by relating respiration rate to a measure of mitochondrial content. Five of the six subjects showed an increase in CS activity but statistical significance was not reached ($p = 0.06$). This likely reflects a type 2 error due to the small number of subjects. While mitochondrial volume may also be increased by rhEpo treatment, several independent measures indicate that mitochondrial capacity can be up-regulated independent of changes in mitochondrial volume. The ratios of OXPHOS (P/COX) and ETS (E/COX) to COX activity were significantly elevated, and the OXPHOS capacity expressed per CS activity showed the same directional pattern (0.84 ± 0.06 vs. 0.73 ± 0.07 , $p = 0.07$) following rhEpo treatment (Table 2) suggesting a higher mitochondrial respiratory capacity per mitochondrion.
- In summary, the present results demonstrate that rhEpo treatment induces increases in both blood hematocrit and skeletal muscle mitochondrial respiratory capacity in humans. Proportional increases in OXPHOS with complex I and I + II substrates were found along with a higher electron transport capacity. The present findings extend on previous studies in rodents to demonstrate an upregulation of human skeletal muscle mitochondrial capacity after rhEpo treatment. The findings may have clinical implications for patients with impaired cardiovascular and muscle metabolic disorders.

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